BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Environmental Microbiology

Cultivated bacterial diversity associated with the carnivorous plant *Utricularia breviscapa* (Lentibulariaceae) from floodplains in Brazil

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ARTICLE INFO

Article history:
Received 18 September 2017
Accepted 24 December 2017
Available online 31 March 2018

Associate Editor: Fernando Andreote

Keywords:
Utricularia breviscapa
Microbial ecology
Aquatic microbiota
Microbial communities

ABSTRACT

Carnivorous plant species, such as *Utricularia* spp., capture and digest prey. This digestion can occur through the secretion of plant digestive enzymes and/or by bacterial digestive enzymes. To comprehend the physiological mechanisms of carnivorous plants, it is essential to understand the microbial diversity related to these plants. Therefore, in the present study, we isolated and classified bacteria from different organs of *Utricularia breviscapa* (stolons and utricles) and from different geographic locations (São Paulo and Mato Grosso). We were able to build the first bacterium collection for *U. breviscapa* and study the diversity of cultivable bacteria. The results show that *U. breviscapa* bacterial diversity varied according to the geographic isolation site (São Paulo and Mato Grosso) but not the analyzed organs (utricle and stolon). We reported that six genera were common to both sample sites (São Paulo and Mato Grosso). These genera have previously been reported to be beneficial to plants, as well as related to the bioremediation process, showing that these isolates present great biotechnological and agricultural potential. This is the first report of an Acidobacteria isolated from *U. breviscapa*. The role of these bacteria inside the plant must be further investigated in order to understand their population dynamics within the host.

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https://doi.org/10.1016/j.bjm.2017.12.013

Introduction

There are over 700 reported species of carnivorous plants distributed in 5 orders (Caryophyllales, Ericales, Lamiales, Oxalidales and Poales) and 10 families that occur in different habitats all over the world.[1] This carnivorous syndrome has arisen independently at least six times in the evolutionary history of angiosperms over an extensive evolutionary timespan and involving different morphological adaptations to capture and digest the prey.[2,3]

The plant must present certain features to be classified as carnivorous, such as actively attracting, capturing and digesting prey, absorbing nutrients from it and obtaining some advantages in growth or reproduction.[4] The prey can be an additional source of N, P, S, K and Mg and complement these photoautotrophic plants.[5,6]

The genus *Utricularia* (Lentibulariaceae), with over 220 species, is the most widespread of the carnivorous plants. One species of this genus is *Utricularia breviscapa*, which can be naturally found in the Antilles and South America. It is an aquatic plant, and its suspended structures contain air-filled parenchyma that allows it to float. Its stolons are threadlike, and bear segmented leaves and utricules (the traps).[7] *Utricularia* species present foliar structures called utricles, which are highly specialized traps that are capable of capturing prey by suction through the action of negative internal hydrostatic pressure.[8,9]

In many carnivorous plant species, digestion does not necessarily occur through the secretion of plant digestive enzymes but is performed by bacterial and/or fungal digestive enzymes.[2,10] For example, in some species of carnivorous plants, such as *Byblis* (Byblidaceae), *Brocchinia* (Bromeliaceae), *Darlingtonia*, *Heliamphora* and some species of *Sarracenia* (Sarraceniaceae), bacterial enzymes are essential in the absence of plant enzyme secretion, and among the plants that have such glands, the bacterial community also plays an important role in the optimization process of prey degradation.[4,11]

These endophytes living inside the plant have innumerous advantages, since the internal tissues provide a stable environment without ultraviolet rays, temperature fluctuations or nutrient competition with other microorganisms as well as an increased availability of nutrients.[12–14] This microorganism-plant interaction is important for the survival of the plant, since these microorganisms represent several benefits for the host plant, providing protection against pathogens, promoting growth, increasing the ability to capture trace elements and several other benefits that are extremely important for the maintenance of plant balance.[15,16]

Therefore, it is important to understand the microbial diversity related to carnivorous plants, particularly bacterial diversity, since it can represent approximately 58% of the total viable microbial biomass,[17] playing a key role in the trap. Caravieri et al.[18] were the first to report the total bacterial diversity from *Utricularia hydrocarpa* and *Genlisea filiformis* traps using a non-cultivable approach, showing that only 1.2% of the observed operational taxonomic units (OTU) were shared by both analyzed plants (*U. hydrocarpa* and *G. filiformis*). Moreover, in addition to plant genotype, trap age can also influence the microbial communities inside the vesicles.[19]

The present study aims to isolate the bacterial community from different organs (stolons and utricles) of *U. breviscapa*, which may have great biotechnological potential, in order to build the first bacterium collection for *U. breviscapa* and study the diversity of cultivable bacteria, aiming to understand their function in this unique environment.

Materials and methods

Plant sampling

The samples of *U. breviscapa* plants were collected from two different floodplains in Brazil: (1) Santo Antônio de Leverger municipality, Mato Grosso state (MT) and (2) Rio Tietê flood plain, Mogi das Cruzes municipality, São Paulo state (SP) (Table 1 and Fig. 1). The sample plants were stored in plastic bags duly identified with sample number and the point from which they were collected, maintained in freshwater at environmental temperature. After collection, the samples were transported to the Laboratory of Molecular Biology and Microbial Ecology where the isolation was immediately carried out. Vouchers are deposited in Herbarium HUMC.

Bacterial isolation

The maceration method was adapted from Kuklinsky-Sobral et al.[20] for the bacterial isolation from weighed stolons and utricles of *U. breviscapa* previously washed in sterilized water. Bacteria were isolated by soaking 5 stolon fragments (approximately 5 mm per fragment) or 10 utricles in phosphate-buffered saline (PBS) containing Na_2HPO_4 ($1.44\,g\,L^{-1}$), KH_2PO_4 ($0.24\,g\,L^{-1}$), KCl ($0.20\,g\,L^{-1}$), and NaCl ($8.00\,g\,L^{-1}$), adjusting the pH to 7.4, and macerating. After maceration, the sample volume was adjusted to 1 mL, appropriate dilutions were carried out and plated onto 10% trypticase soy agar (TSA) supplemented with $50\,mg\,mL^{-1}$ of the fungicide Imazalil (Magnate 500 CE, Agricur), and the plates were incubated at 28 °C for 2–15 days. The colonies were removed from the plates, inoculated onto 10% TSA agar medium, incubated at 28 °C for 2–10 days, and then each isolate was suspended in a 20% glycerol solution and stored at −70 °C.

Table 1 – Sampling locations and characteristics.

General location	City	Coordinates	River/lake	Characteristics
Mato Grosso state	Santo Antônio de Leverger	S15°59′31.8″ W55°48′57.4″	River Aricá floodplain	Lentic environment, low depth
São Paulo state	Mogi das Cruzes	S23°31′58.5″ W46°08′38.3″	River Tietê floodplain	Lentic environment, medium depth

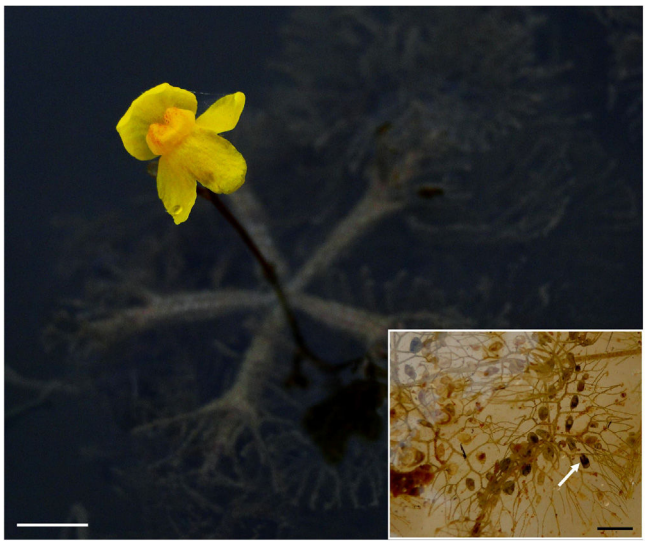

Fig. 1 – Habit of *Utricularia breviscapa* with inflorescence (bar = 10 mm). The detail shows a stolon with utricles (arrow indicates an utricule; bar = 5 mm).

Statistical analyses were carried out with biological triplicates for each treatment for bacterial quantification in the isolation experiments, which were performed in a completely random design. The significance of the observed differences was verified using a new analysis of variance ($p = 0.05$). Analyses were conducted using R software version 3.0.1.

Polymerase chain reaction (PCR) amplification and 16S rDNA sequencing

After cultivation, the bacterial DNA was extracted according to Araújo et al.[21]. A partial sequence of the 16S rRNA gene was amplified using the pair of primers R1378[22] and P027F.[23] PCRs were performed according to Dourado et al.[24] All PCR amplification was checked through electrophoresis on agarose gel (1.5%, w/v agarose) and UV visualization of the ethidium bromide-stained gels, after which the PCR products were purified using a GFX PCR DNA and gel band purification kit (Amersham Biosciences) and sequenced by Sanger Sequencing Technology[25] using the primer 1378R.

Analysis of the 16S rRNA gene sequence

Sequences were obtained from the bacteria isolated from *U. breviscapa* stolons and utricles from São Paulo and Mato Grosso state. Prior to the analysis, all chromatograms were trimmed with Phred-Phrap (http://www.phrap.com/phred/). The sequences were clustered as operational taxonomic units (OTU) using MOTHUR[26] and a cut-off of 97% identity and further examined using rarefaction analysis and Libshuff, and dendrograms were constructed. Furthermore, richness and diversity were also calculated using MOTHUR based on non-parametric richness (Ace and Chao1) and diversity (Shannon-Weaver and Simpson) indexes with cut-offs for similarity at 97% (0.03), 95% (0.05) and 91% (0.09) identity. Taxonomic classifications were performed using RDP Query (https://rdp.cme.msu.edu) as database parameters. For phenetic analysis, the sequences were aligned using ClustalW,[27] and the distance was calculated using the Jukes and Cantor model[28] using Neighbor Joining method in MEGA 5 software.[29] The branches were tested with bootstrap analyses (1000 replications), and the layout of trees was designed using the online application "Interactive Tree Of Life" (iTOL) (http://itol.embl.de/).[30]

A total of 200 DNA sequences of partial 16S rRNA genes were deposited in the GenBank database under accession numbers KY453794 to KY453980.

Results and discussion

A total of 200 sequences were obtained from bacteria isolated from *U. breviscapa*. Higher isolated bacterial density was observed in association with plants from Mato Grosso (MT) (Fig. 2). The bacterial density averages from MT samples were 4.9×10^5 CFU utricle^{-1} and 3.6×10^7 CFU g of stolon^{-1}, while the samples from São Paulo (SP) had bacterial densities of 6.0×10^4 CFU utricle^{-1} and 1.2×10^7 CFU g of stolon^{-1} (Fig. 2).

The richness estimator of OTUs performed with the Chao1 and Ace indexes at 97% (Table 2) showed a greater bacterial richness in SP plants than in MT plants. According to the Chao1 parameter, we can observe the highest richness in utricles followed by stolons in SP plants. However, based on the Ace estimator, the opposite was observed, in which the Ace index showed the highest richness in stolons followed by

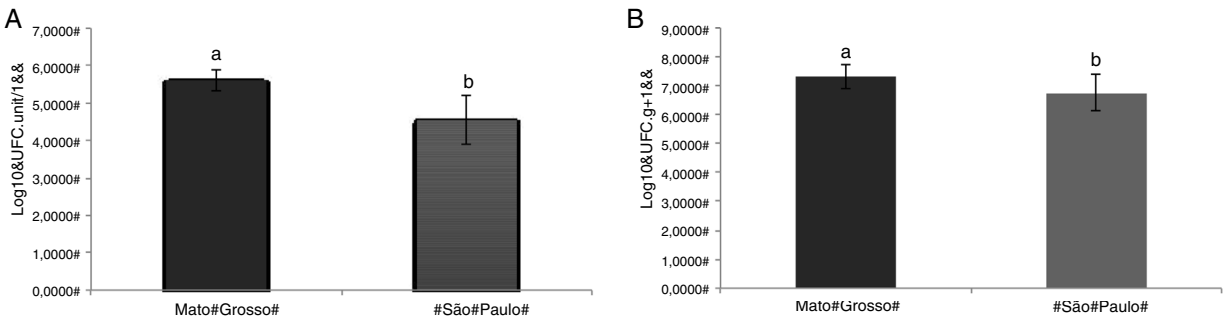

Fig. 2 – Bacterial density in (A) utricles and (B) stolons of *U. breviscapa* from Mato Grosso (MT) and São Paulo (SP). Statistically different at $p < 0.05$.

Table 2 – Diversity index and richness estimation of analyzed OTUs.

Library	Similarity	OTUs	Chao-1	Ace	Shannon (H)	Simpson (1-D)
			Richness estimator		Diversity indexes	
utricSP	97%	37	107 (62–236)	104 (63–211)	3.2 (3.0–3.5)	0.95 (0.98–0.93)
utricMT	97%	25	56 (34–124)	66 (38–152)	2.5 (2.1–2.9)	0.85 (0.94–0.76)
stolSP	97%	34	93 (55–196)	228 (144–373)	3.3 (3.1–3.6)	0.97 (0.99–0.95)
stolMT	97%	17	56 (27–168)	82 (46–163)	2.64 (2.3–2.9)	0.94 (0.99–0.90)

utricles. In terms of bacterial richness in MT plants, Chao1 estimates a statistically equal richness in both utricles and stolons (Table 2).

It is common knowledge that the performance of non-parametric estimators depends on the species abundance distribution in the sample, and the preference for one over another is a difficult issue. Basualdo[31] observed in his work that the Ace index was better when the observed richness was low and Chao1 when the observed richness was high. In another study, Hortal et al.[32] mentioned that abundance-based non-parametric estimators (Ace and Chao1) are more precise compared with other richness estimators; however, their precision diminishes at lower sampling intensities, producing less consistent results. This suggests that the choice among richness estimator parameters can vary between sampling characteristics, even in the same study.

According to the Simpson index diversity at 97%, a higher diversity of OTUs was found in libraries constructed from SP plants, with the highest diversity in stolons (stolSP) followed by utricles (utricSP). Similar to São Paulo, the bacterial diversity index in MT plants was also higher in stolons (stolMT) followed by utricles (utricMT). The Shannon diversity index at 97% showed the same result.

Therefore, for all indexes used (Chao1, Ace, Simpson and Shannon), higher diversity rates were observed in plants from São Paulo. There is no apparent reason why São Paulo samples present a higher diversity when compared with Mato Grosso samples, but it is known that there are various characteristics that could be considered to contribute to this variation, of which the trap age has been the most cited in the literature, but the quality of water can also impact the microbial diversity. Plachno et al.[19] found that old traps were colonized by attached bacteria, but they can also be found freely suspended in trap fluid, as shown in Sirová et al.[17] However, determining utricle dynamics is further complicated by the rapid aging of traps (or pitcher), as their life cycle is completed over approximately 30 days, and many environmental changes occur during this time.[33] Although their life cycle is short, the interior of utricles presents low concentrations of oxygen, and it has been postulated that the organisms trapped by *Utricularia* die as a result of oxygen depletion.[34] This anoxic environment is reflected in its commensal communities, selecting for anaerobic and facultatively aerobic bacteria.[35] Pitsch et al.[36] reported a ciliate commensal that uses algae to circumvent the low concentration of oxygen inside the utricles. Furthermore, some researchers have assumed that this high abundance of commensal organisms also occurs in empty traps; in the same way, these microorganisms are not specialized for living inside the traps, and they can therefore live either on the external surface or freely as plankton in the ambient water.[37]

Table 3 – Libshuff analysis comparing coverage between libraries.

Comparison	Score	Significance
stolMT-stolSP	0.006	0.0044
stolSP-stolMT	0.025	<0.0001
stolMT-utricMT	0.010	0.0015
utricMT-stolMT	0.053	<0.0001
stolMT-utricSP	0.011	0.0037
utricSP-stolMT	0.069	<0.0001
stolSP-utricMT	0.029	<0.0001
utricMT-stolSP	0.009	<0.0001
stolSP-utricSP	0.001	0.5787
utricSP-stolSP	0.006	0.0009
utricMT-utricSP	0.005	0.0001
utricSP-utricMT	0.048	<0.0001

Another factor that could influence the microbial community inside the traps is the composition of the water surrounding the trap that will be sucked in together with the prey or even attached to the prey. Thus, the bacterial community is selected according to the physical–chemical conditions inside the trap. It is important to emphasize that the two sampled sites were located in floodplain areas. River floodplain systems are known for their heterogeneity in habitats and hydrological pulses, presenting great temporal and spatial variation in their limnological conditions, which, in turn, should influence the bacterial community composition.[38]

The Libshuff significance test indicated that those libraries belonging to the same part of the plant (stolSP/stolMT and utricSP/utricMT) (Table 3) differ significantly for each sampled site (São Paulo and Mato Grosso). Moreover, although

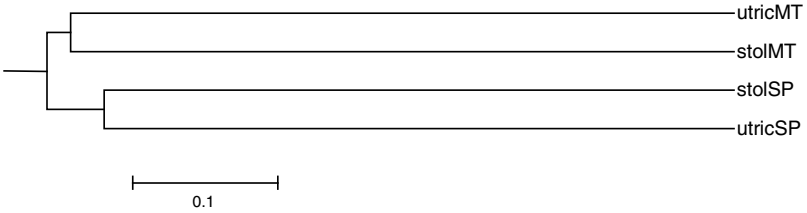

Fig. 3 – Dendrogram of bacterial communities associated to *U. breviscapa*.

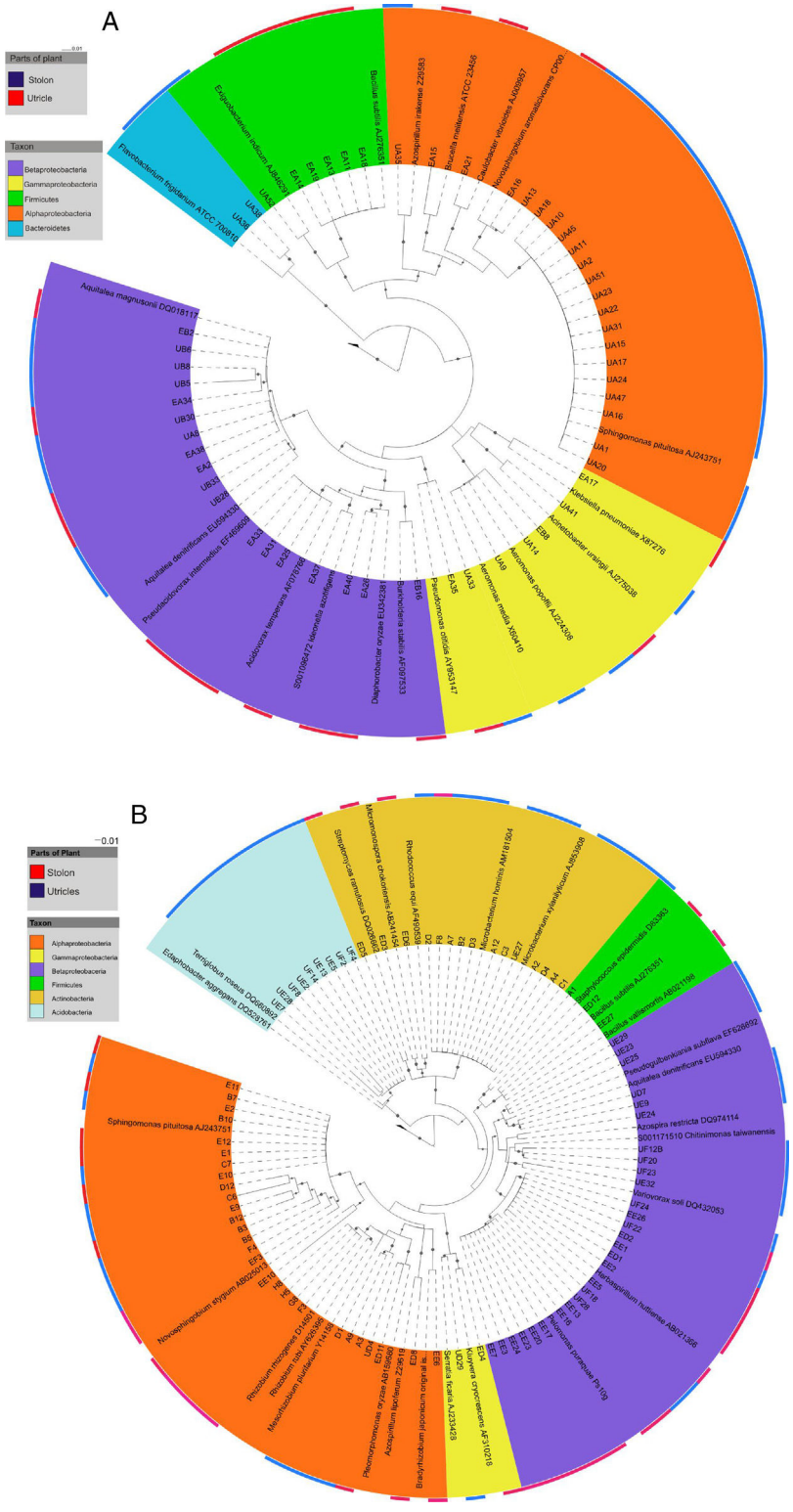

Fig. 4 – Phenetic trees representing the cultivable bacterial found in utricles and stolons from *U. breviscapa* were built using Neighbor Joining method in MEGA 5 software. (A) Isolates from Mato Grosso and (B) from São Paulo. The solid gray circles next to the tree branches correspond to bootstrap values higher than 50%. *Brachyspira innocens* (S000437178); *Brachyspira hyodysenteriae* (S000437188), *Brachyspira hyodysenteriae* (S000437188) and *Brachyspira hyodysenteriae* (S000437188) was used as outgroup.

they are associated with the same site, the significance analysis for utricleMT and stolonMT shows a significant difference between these parts of the host plant. On the other hand, when comparing utricleSP and stolonSP, which present higher richness and diversity in their 16S rDNA libraries, do not vary, indicating that only in less diverse environments are there different colonization patterns between stolons and utricles.

A dendrogram of bacterial communities showed similarities between the same site libraries (Fig. 3). Agreeing with the richness and diversity indexes, the libraries were grouped according to the sampled site, grouping utricles and stolons from São Paulo (utricSP and stolSP) in the same branch (with presents higher diversity), while utricles and stolons from Mato Grosso (utricMT and stolMT) were grouped in another branch (which presents lower diversity). This indicates that there is greater bacterial community similarity between different parts of the same host plant collected from the same sampled site (São Paulo or Mato Grosso), corroborating the hypothesis that the place of origin plays the main role in bacteria-plant interactions, since the environment supplies the microbial diversity to the plant host.

In looking at the 200 isolates from SP and MT, respectively, the 16S rRNA analysis of plant isolates from MT showed 22 bacterial genera, among them *Sphingomonas* (36.71%), *Aquitalea* (18.99%) and *Bacillus* (6.33%), while in the plant isolates from São Paulo, we found 31 bacterial genera, of which the predominant genera were *Microbacterium* (20.66%), *Sphingomonas* (14.05%) and *Pelomonas* (9.09%) (Fig. 4). Caravieri et al.[18] also found *Sphingomonas* and *Microbacterium* genera in association with *U. hydrocarpa*, but the culture-independent methods used in their work found these OTUs among the less abundant genera.

Six genera were common to both sampled sites (SP and MT): *Aquitalea*, *Azospirillum*, *Bacillus*, *Chromobacterium*, *Novosphingobium* and *Sphingomonas* (Figs. 4 and 5). As shown for bacterial density, it was also verified that the abundance of cultivable bacterial species varied according to the sampled sites, suggesting that the plant selects the bacterial community in its tissues, and this selection must occur from the diversity present in its surrounding environment. Moreover, Koopman et al.[39] observed that the bacterial community within the traps of *Sarracenia alata* plants was significantly different from the soil surrounding the plant and varied over time, suggesting that the community associated with the plants should not occur randomly but should be dependent on the interaction between the plant genotype and the environmental conditions.

The *Utricularia* samples were taken from muddy and dirty water, where the most abundant genera of both studied sites belonged to the *Sphingomonas* genus, which has been previously reported to colonize different polluted environments, both aquatic and terrestrial, being able to use polycyclic aromatic hydrocarbons as its sole source of carbon and energy.[40] Moreover, this genus has been reported to have a beneficial interaction with plants, producing phytohormones such as gibberellins and indole acetic acid, promoting the growth of *Solanum lycopersicum*,[41] and producing exopolysaccharides,[42,43] which have also been reported to be a factor in plant–bacterium interactions for *Paenibacillus polymyxa*.[44]

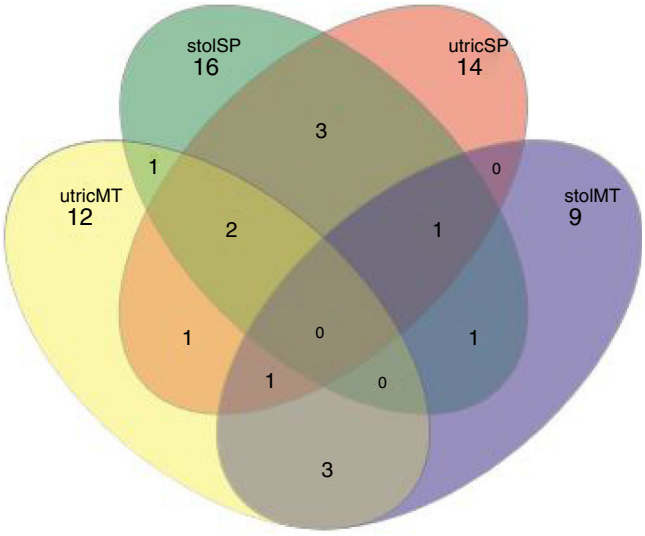

Fig. 5 – Venn diagram represented by shared UTO's of stolons (stol) and utricules (utric) of *U. breviscapa* from Mato Grosso (MT) and São Paulo (SP) (dissimilarity of 0.09). Therefore: utricMT: utricules from Mato Grosso, stolSP: stolons from São Paulo, utricSP: utricules from São Paulo and stolMT: stolons from Mato Grosso.

In the present work, the genus *Aquitalea* was one of the most abundant genera associated with utricles from sampled SP and MT *Utricularia*. The genus *Aquitalea* was described in 2006 by Lau et al.,[45] from a facultative anaerobic bacterium strain living in lakes. This genus is most commonly found in humic and oligotrophic lakes and in other water sources.[46] Woo et al.[47] showed that the genus *Aquitalea* was one of the most abundant genera isolated from wet tropical forest soils, an environment rich in organic matter to decompose, presenting high levels of enzyme activities. Although little is known about the mechanism of digestion in *Utricularia*, evidence has been raised for the presence of enzymatic activity inside the trap, and a considerable proportion of the enzymatic activity in the trap fluid is assumed to be derived from the bacterial community.[48]

Other abundant genera associated with *Utricularia* from SP were *Pelomonas* and *Microbacterium*, which have also been reported as beneficial bacteria to host plants. *Pelomonas* spp. isolated from industrial water or from humic and oligotrophic lakes are able to fix nitrogen and reduce nitrate[45,49]; they can also be involved in the bioremediation process because they degrade aromatic compounds. *Microbacterium* isolated endophytically and from the plant rhizoplane are also able to fix nitrogen,[50] solubilize phosphate, oxidize sulphur, reduce nitrate and produce both indole acetic acid (IAA) and ACC deaminase (associated with stress reduction in plants by the inhibition of ethylene synthesis), showing their potential to promote plant growth.[51]

The *Bacillus* spp. found in both libraries (SP and MT) also have plant beneficial characteristics, such as the presence of the nitrate reductase enzyme and the potential to inhibit several phytopathogenic fungi (for example, *Alternaria alternata*, *Cryphonectria parasitica*, *Fusarium graminearum*, *Phytophthora*

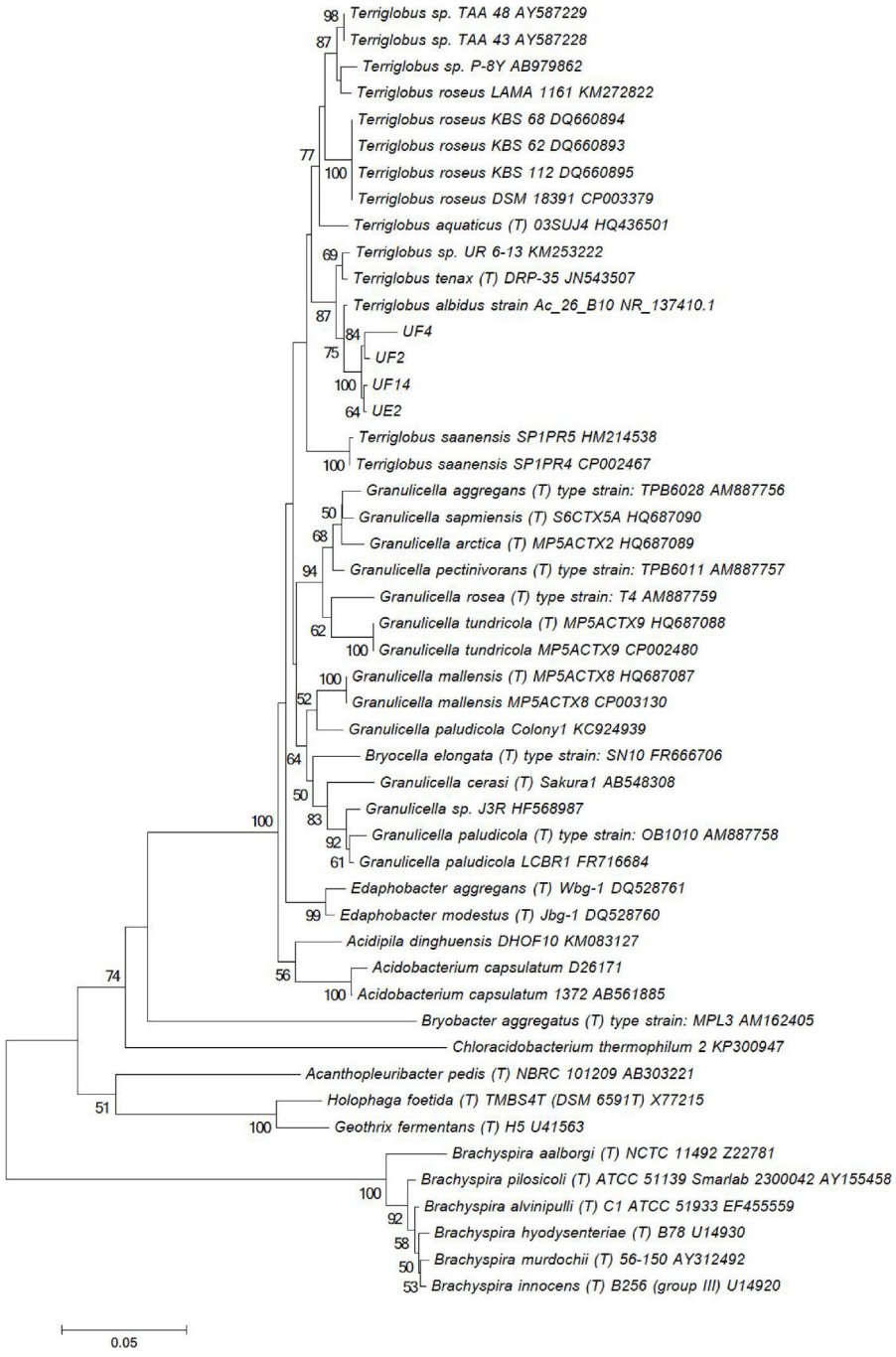

Fig. 6 – Phenetic tree representing the cultivable Acidobacteria found in association with utricles of *U. breviscapa* from SP using Neighbor Joining method in MEGA 5 software. Numbers above the branches indicates bootstrap values. *Brachyspira* species were used as outgroup.

capsici and *Rhizoctonia solani*), demonstrating their important role in host plant defense.[52]

Furthermore, SP plants presented exclusive groups, such as the Actinobacteria that were isolated from stolons and utricles, confirming the specificity dependent on the geographic location from which the plants were collected. However, despite presenting similar libraries with no difference in the Libshuff significance test, plant organs can also select a bacterial group, such as the phyla Acidobacteria and Bacteroidetes, which were found only in utricles from plants collected in São Paulo and Mato Grosso, respectively (Fig. 4). These results support the hypothesis that plants select the bacterial communities associated with stolons and utricles based on the available community present in their surrounding environment.

Another important point we need to address is the utricle isolates that belong to the Acidobacteria group, identified as the genus *Terriglobus albidus* (99% similarity), a genus of fastidious growth (Fig. 6). Phylum Acidobacteria is one of the most abundant in soils, and its members have been found by

cultivation independent methods in many types of environments. Acidobacteria is known to be abundant in trap fluid, and some species are known to live in acidic environments.[53] Sirová et al.[17] showed that the trap fluid pH is low in *U. vulgaris*, ranging from pH 4.2 to 5.1 according to the trap age, and this could be correlated with the phosphatase activity inside the trap.

However, members of the Acidobacteria group have proven difficult to isolate and cultivate under laboratory conditions, and there are still few species with valid names, and few are well defined; among them are members of the genera *Acidobacterium*, *Acanthopleuribacter*, *Bryobacter*, *Edaphobacter*, *Geothrix*, *Granulicella*, *Holophaga* and *Terriglobus*.[54,55] *Terriglobus* sp. was previously isolated from water[56] and from specific soil conditions, such as desert soils,[57] arctic tundra soils[58] and the rhizosphere of a medicinal plant.[59] *T. albidus* was described in 2015[60] isolated from a semiarid savannah soil collected in northern Namibia, however the present work present the first report of cultivable bacteria belonging to this genus isolated in association with a carnivorous plant.

Caravieri et al.,[18] using culture-independent methods, reported that *Acidobacterium* was one of the dominant genera in association with *U. hydrocarpa*. There are advantages in retrieving an *Acidobacterium* isolate due to its biotechnological applications, such as the production of an extracellular matrix that apparently facilitates the flocculation of cells in liquid media.[61] Therefore, our research group is currently investigating the biotechnological potential of these *Acidobacterium* isolates.

Conclusions

The results show that the bacterial diversity of *U. breviscapa* varied according to the isolation location (São Paulo and Mato Grosso) rather than organs. This is also the first report of an Acidobacteria (*Terriglobus* genus) isolated in association with utricles of *U. breviscapa*. The role of these bacteria inside the utricles and stolons must be further investigated in order to understand their population dynamics within the host plant.

Conflicts of interest

Declarations of interest: none

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